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Recombinant Duck Interferon: A New Reagent for Studying the Mode of Interferon Action against Hepatitis B Virus

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Although interferon is widely used to treat chronic hepatitis B virus infections, its mode of action against hepadnaviruses is largely unknown. This deficit is due mainly to the lack of suitable model systems. The duck system could not be used because purified duck interferon was not available in sufficient quantities. We have now cloned a DNA fragment that contains an intronless gene for duck interferon. The primary translation product consists of 191 amino acids, the N-terminal 30 residues of which constitute a signal peptide. Mature duck interferon is 50% identical to the recently cloned chicken interferon. Sequence homology to mammalian interferons is marginal, but conservation of four cysteine residues and inducibility by virus indicate a distant relationship between duck interferon and mammalian type I interferons. Purified recombinant duck interferon from *Escherichia coli* is biologically active: it activates the interferon-inducible Mx gene, prevents cell destruction by cytolytic RNA viruses, and has a strong inhibitory effect on duck hepatitis B virus in cultured primary duck hepatocytes. This new reagent should help to define the interferon-sensitive step of the hepadnavirus life cycle. Furthermore, the duck system can now be used for systematic studies of the *in vivo* effectiveness of interferon in chronic hepatitis B virus infections. © 1995 Academic Press, Inc.

INTRODUCTION

Members of the Hepadnavirus family exhibit a pronounced tissue and species specificity. Chronic infections with human hepatitis B virus (HBV) lead to severe liver damage, cirrhosis, and hepatocellular carcinomas in a large number of patients (Israel and London, 1991). Interferon-α (IFN-α) has been shown to be effective in inducing remissions of chronic viral hepatitis. Although 40% of the patients benefit from IFN treatment, a significant number of patients are refractory to the antiviral effects of IFN (Perrillo *et al.*, 1990; Hoofnagle, 1992; Saracco *et al.*, 1994). Since the IFN therapy is effective in only selected patients, a systematic analysis of factors which might influence the clinical outcome of the IFN treatment is required. However, progress in this direction is slow due to the lack of a suitable experimental system. The duck hepatitis B virus (DHBV) model has been of great value for many aspects of the molecular biology of the hepadnaviruses (Mason *et al.*, 1980; Tuttleman *et al.*, 1986; Schödel *et al.*, 1991), but studies on the interferon effect were severely hampered by the unavailability of recombinant duck IFN (DuIFN). With the cloning of a DuIFN gene and the successful production of recombinant DuIFN in *Escherichia coli* reported in this paper, these limitations no longer exist.

The IFNs of birds are rather poorly characterized. Although the historical experiments which led to the discovery of IFN were performed with embryonated chicken eggs (Isaacs and Lindenmann, 1957), the first gene for chick IFN was cloned only very recently (Sekellick *et al.*, 1994) and analysis of the chicken IFN system at the molecular level is just beginning (Schultz *et al.*, 1995b). Available data show that there is little sequence conservation between avian and mammalian IFNs (Sekellick *et al.*, 1994). Nonetheless, the cloned chick IFN clearly is a type I IFN, like the mammalian IFNs-α, β, ω, and τ: chick IFN is induced in response to virus, it shares many physicochemical properties with mammalian type I IFNs, and it strongly activates the type I IFN-responsive promoter of the chicken Mx gene (Schultz *et al.*, 1995a). Unlike mammalian IFN-γ, the cloned chick IFN lacks intrinsic macrophage-activating factor activity (Schultz *et al.*, 1995a), further supporting the view that it is a type I IFN. The biochemical characterization of natural DuIFN is rudimentary: it was described as an antiviral activity of 17.5–20 kDa secreted by virus-infected duck embryo fibroblasts (Ziegler and Joklik, 1981). Natural DuIFN has little or no cross-reactivity on chicken cells (Ziegler and Joklik, 1981).

We have now cloned an intronless gene that codes for a virus-induced DuIFN. The primary translation product consists of 191 amino acids, the N-terminal 30 residues

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of which constitute a signal peptide. A polypeptide corresponding to the mature DuIFN sequence was expressed in *E. coli* and purified to homogeneity. Recombinant DuIFN had a strong antiviral effect toward several RNA viruses and was a potent inhibitor of DHBV in primary duck hepatocytes.

MATERIALS AND METHODS

Cell culture

Duck embryo cells were prepared from 10-day-old embryos from wild ducks (*Anas platyrhynchos*) by trypsinization. They were maintained in Dulbecco's modified minimal essential medium (DMEM) supplemented with 10% fetal calf serum (FCS). The duck embryo cell line (DEC141; ATCC CCL141) and COS7 cells were propagated in DMEM containing 10% FCS. CEC-32 cells were cultured in DMEM supplemented with 8% FCS and 2% chicken serum (Kaaden *et al.*, 1982). Primary duck hepatocytes were prepared as described previously (Köck and Schlicht, 1993). Briefly, fertilized duck eggs were incubated for at least 18 days in an automatic breeding incubator at 37.6° and 50 to 60% humidity. The livers were collected and treated for 20 min with 0.5% collagenase (Sigma) in 5 ml of Williams E medium (GIBCO) supplemented with 2 m*M* L-glutamine, 15 m*M* HEPES buffer (*N*-2-hydroxyethylpiperazine-*N*-2-ethanesulfonic acid, pH 7.3), 100 U per milliliter penicillin, 100 μg per milliliter streptomycin, 10^{-5} *M* hydrocortisone (Sigma), 1 μg per milliliter insulin (Sigma), and 1.5% dimethyl sulfoxide (Sigma). After washing three times with medium, the cells of one liver were resuspended in 24 ml of fresh medium and seeded into six-well dishes (1 ml per well).

Viruses

The influenza A virus strain FPV-B and Newcastle disease virus (NDV) strain H53 were grown in the allantoic cavity of 10-day-old embryonated chicken eggs. Vesicular stomatis virus (VSV; serotype Indiana) was prepared in 3T3 cells (Pavlovic *et al.*, 1990). Serum of DHBV-positive ducks served as a stock for infectious DHBV (Köck and Schlicht, 1993).

Genomic Southern blots

Samples of DNA (20 μg) from duck embryo cells were digested with various restriction enzymes, and the fragments were size-fractionated by electrophoresis through a 1% agarose gel and transferred to a nylon membrane in 0.4 *M* NaOH by standard techniques (Ausubel *et al.*, 1992). Hybridization was carried out in 0.12 *M* sodium phosphate, pH 7.3, 0.25 *M* NaCl, 7% SDS, 1 m*M* EDTA, 20% formamide, and 200 μg per milliliter of denatured herring sperm DNA. A nick-translated fragment of the chicken IFN cDNA (Sekellick *et al.*, 1994) served as hybridization probe.

Isolation of a DNA fragment containing the DuIFN gene

DNA from duck embryo cells was restricted with *Hin*dIII and size-fractionated by electrophoresis through a 1% low-melting-point agarose gel. DNA fragments of approximately 2.7 kb length were isolated from the gel and ligated into the cloning vector pBluescript (SK) (Stratagene). The ligation mixture was used to transform *E. coli* strain XL1-Blue MRF′. Ampicillin-resistant colonies were screened by colony hybridization using a radiolabeled fragment of the chicken IFN cDNA as a probe.

DNA sequence analysis

Nucleotide sequences were determined by the dideoxy chain determination method using the T7 polymerase kit from Pharmacia.

Northern blot assays

Duck embryo cells were either treated with UV-inactivated NDV for 8 hr as previously described (Bazzigher *et al.*, 1992) or with 10 ng per milliliter of recombinant DuIFN for 6 hr before total RNA was prepared (Chomczynski and Sacchi, 1987). RNAs (20 μg per lane) were separated by electrophoresis through agarose gels containing formaldehyde before they were blotted onto nitrocellulose. The blots were hybridized with radiolabeled probes derived from plasmids containing DuIFN or duck Mx sequences.

Plasmid constructions

PCR techniques were used to generate a prokaryotic expression construct for DuIFN that lacks the N-terminal signal peptide sequences. Primer 1 (5′AAGTACATATGTGCAGCCCCCTGCGCCT3′) corresponds to nucleotide positions 1483 to 1499 of the cloned DNA fragment and introduces an additional *Nde*I restriction site. Primer 2 (5′AGATAGGATCCTTAGCGCATGGTGCG3′) is reverse complementary to nucleotide positions 1954 to 1968 and provided a new *Bam*HI site that facilitated subsequent cloning experiments. After restriction with *Nde*I and *Bam*HI, the PCR product was cloned into the corresponding sites of pET3a (Studier and Moffat, 1986).

For expression of recombinant DuIFN in COS7 cells, a PCR fragment was generated with primer 2 and primer 3(5′GATATCGAATTCAACAGGCTCTTGC3′), corresponding to nucleotide positions 1357 to 1370 of the cloned DNA fragment (Fig. 3). The PCR product which contains the entire open reading frame of DuIFN was cloned into the eukaryotic expression vector pcDNAI (*invitrogen*).

Purification of recombinant DuIFN from *E. coli*

DuIFN synthesis from the pET expression construct was induced by adding 0.5 m*M* isopropyl β-D-thiogalac-

topyranoside (IPTG) to the *E. coli* culture medium as described (Schultz *et al.*, 1995b). The bacterial pellet from a 1-liter culture was suspended in 15 ml of buffer A (50 m*M* Tris–HCl, pH 8.0, 7.5% glycerol, 0.1 m*M* EDTA, 1 m*M* DTT) supplemented with 50 m*M* NaCl and the cells were lysed by the addition of 1.5 ml of buffer B (50 m*M* EDTA, pH 8.0, 10% Triton X-100, 2.5 mg/ml of lysozyme, 0.05 m*M* PMSF) and incubation on ice for 1 hr. After the addition of 5 m*M* $MgCl_2$, the suspension was sonicated seven times for 30 sec. The solid material was collected by centrifugation and the supernatant was discarded. Solubilization of the pellet fraction was achieved by incubation in 15 ml of buffer A containing 6 *M* guanidine hydrochloride for 30 min on ice. The insoluble material was removed by centrifugation and the cleared supernatant was dialyzed against buffer A supplemented with 50 m*M* NaCl, 0.05 m*M* PMSF, 0.5 m*M* DTT, and continuously decreasing amounts of guanidine hydrochloride over a period of 48 hr. Precipitated material was removed by centrifugation before the supernatant was subjected to Q-Sepharose column chromatography (50 m*M* Tris–HCl, pH 7.8, 7.5% glycerol, 50 m*M* NaCl). The flowthrough was collected and the buffer was exchanged on a PD10 column (Pharmacia) before it was applied to a MonoS column equilibrated with 50 m*M* Na-phosphate, pH 7.6, and 7.5% glycerol. Proteins that bound to the matrix were eluted with a 20-ml linear gradient of NaCl (0 to 1000 m*M*) at a flow rate of 0.5 ml/min. DuIFN eluted at 400 to 450 m*M* NaCl.

A modified procedure for purifying DuIFN was as follows: the insoluble bacterial pellet was dissolved in 15 ml buffer A* (200 m*M* Tris–HCl, pH 7.8, 7.5% glycerol, 0.1 m*M* EDTA, 0.3% Triton X-100) containing 6 *M* guanidine hydrochloride and 1 m*M* DTT. Dialysis was carried out in buffer A* supplemented with 50 m*M* NaCl and 0.5 m*M* DTT. Following Q-Sepharose column chromatography, the flowthrough was applied directly to a Fractogel EMD SO_3^- column. DuIFN was eluted with a 20-ml linear gradient of NaCl.

The optimized protocol for purifying DuIFN of high specific activity is described below. The bacterial pellet from a 1-liter culture was sonicated in 10 ml of sonication buffer (50 m*M* Na-phosphate, 300 m*M* NaCl, 10 m*M* β-mercaptoethanol, pH 7.8). The solid material was collected by centrifugation and solubilized by incubation for 90 min in 10 ml of solubilization buffer (6 *M* guanidine hydrochloride, 100 m*M* NaH_2PO_4, 10 m*M* Tris–HCl, 10 m*M* β-mercaptoethanol, pH 8.0). After centrifugation at 10,000 *g* for 30 min the supernatant was mixed with 5 ml of a 50% slurry of Ni^{2+} chelate agarose (Qiagen), previously equilibrated in solubilization buffer. The slurry was stirred for 90 min at room temperature before it was filled into a column and washed with 20 column volumes of solubilization buffer and 10 column volumes of buffer H (8 *M* urea, 100 m*M* NaH_2PO_4, 10 m*M* Tris–HCl, pH 8.0). Buffer H was gradually exchanged for buffer H* containing 2 *M* urea and 1 *M* NaCl. DuIFN was eluted with a 20-ml linear gradient of elution buffer (A: 50 m*M* Na-acetate, 1 *M* NaCl, 10 m*M* Tris–HCl, 2 *M* urea, 10 m*M* β-mercaptoethanol, pH 6.5; B: 50 m*M* Na-acetate, 1 *M* NaCl, 2 *M* urea, 10 m*M* β-mercaptoethanol, pH 3.6). Fractions containing DuIFN were pooled and dialyzed against 50 m*M* Na-acetate, pH 3.6, containing 50 m*M* NaCl.

Production of recombinant DuIFN in COS7 cells

Transfections of COS cells with the DuIFN expression construct were done as described previously for chicken IFN (Schultz *et al.*, 1995b). At 72 hr posttransfection, the medium of the transfected cultures was harvested and the cell debris were removed by centrifugation (Schultz *et al.*, 1995b).

IFN titrations

The antiviral titer of the various IFN preparations was determined by the cytopathic effect reduction assay (Schultz *et al.*, 1995b) using the duck embryo cell line DEC141 in 96-well microtiter plates. The cultures were stimulated with twofold serial dilutions of DuIFN for 15 hr before challenge with VSV at a multiplicity of 1. IFN titers are expressed as reciprocals of the dilutions that resulted in 50% protection against virus-induced cell destruction. Supernatants of transfected COS cells expressing recombinant DuIFN (25,000 units per milliliter) served as a laboratory standard.

Virus yield reduction assays

To quantitate the antiviral effect of DuIFN, DEC141 cells were treated with DuIFN for 15 hr before infection with influenza virus, VSV, or NDV at a multiplicity of 1. Samples of the supernatants of the infected cultures were removed at 12 and 48 hr postinfection, and the viral titers were determined on CEC-32 cells by the tissue culture infecting dose 50 method (Pavlovic *et al.*, 1990).

IFN treatment of primary duck hepatocytes

For the experiment shown in Fig. 6, cells in six-well dishes were incubated with 100 units per milliliter of DuIFN for 15 hr before infection with 20 μl of DHBV stock per well. The virus inoculum was removed after 5 hr, and the cells were washed once with 50 m*M* glycin buffer, pH 2.2, containing 150 m*M* NaCl and once with medium. Fresh medium containing or lacking DuIFN was then added and the cultures were incubated at 37°. For the experiment shown in Fig. 7, the primary duck hepatocyte cultures were infected with 20 μl of the DHBV stock per well about 20 hr after plating into six-well dishes. The virus inoculum was removed at 3 hr postinfection and replaced by fresh medium. At 24 and 72 hr postinfection, treatment with DuIFN at a concentration of 100 units per milliliter was started. The culture medium was replaced

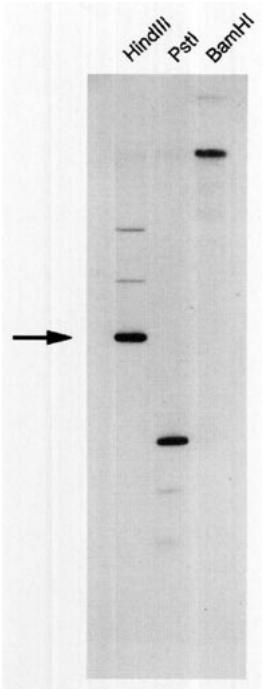

FIG. 1. Southern blot analysis of duck DNA with a chick IFN cDNA probe. DNA (20 μg per lane) was restricted with the indicated enzymes, electrophoresed through a 0.8% agarose gel, and blotted onto a nylon membrane. The membrane was probed with a radiolabeled fragment of ChIFN cDNA that included the entire open reading frame. The arrow marks the strongly hybridizing 2.7-kb *Hin*dIII fragment that was cloned and characterized.

every second day, and IFN was added where appropriate. Parallel cultures of infected hepatocytes were subjected to treatment with 2′,3′-dideoxy guanosine (ddG; Pharmacia) at a concentration of 20 μg per milliliter. The culture medium was replaced every second day, while fresh ddG was added every day.

Analysis of the DHBV DNA content in infected hepatocytes by PCR

Cells were lysed for 2 hr at 56° in 0.3 ml of lysis buffer (50 m*M* KCl, 10 m*M* Tris–HCl, pH 8.3, 0.45% Tween 20, 0.45% Nonidet P-40, 0.1 mg/ml proteinase K) per well of a six-well plate. After this step, the proteinase K was inactivated by incubating the samples for 10 min at 95°. PCR was done with samples of the cell lysates under conditions that guaranteed high selectivity of the primers designed to amplify viral cccDNA (Köck and Schlicht, 1993). The oligonucleotide primer pairs for amplification of cccDNA and total viral DNA were the same as described previously (Köck and Schlicht, 1993). Samples (1/5 of the PCR products) were subjected to Southern blot analysis. Hybridization was carried out with nick-translated full-length DHBV DNA under the conditions described above, except that the hybridization buffer contained 50% formamide.

Nucleotide sequence accession number

The sequence of the genomic DuIFN clone has been assigned EMBL/GenBank Accession No. X84764.

RESULTS

Identification, cloning, and characterization of a DuIFN gene

Southern blot analysis of duck DNA indicated that the previously cloned cDNA for a chicken IFN (ChIFN) (Sekellick *et al.*, 1994) might be a suitable probe for isolating the IFN genes of the duck. At low stringency of hybridization, the ChIFN probe detected a prominent and several minor fragments of duck DNA after restriction with *Hin*dIII, *Pst*I, or *Bam*HI (Fig. 1). To clone the prominently hybridizing 2.7-kb *Hin*dIII fragment, duck DNA was restricted, size-selected by low-melting-point agarose gel electrophoresis, and ligated into a *Hin*dIII-restricted cloning vector. Clones carrying sequences related to ChIFN were identified by colony hybridization. To verify that the cloned fragment contained a DuIFN gene, we tested whether it detected a virus-induced transcript in duck embryo cells. At high stringency of hybridization, this probe recognized an abundant 1.2-kb transcript of NDV-infected cells which was undetectable in uninfected cells (Fig. 2). The sequence of the cloned 2762-bp *Hin*dIII fragment is shown in Fig. 3A. It contains a complete intronless DuIFN gene: an open reading frame that extends from an initiation codon at position 1393 to a termination codon at position 1966 encodes a protein of 191 amino acids. The ATG at the beginning of the ORF conforms to the consensus sequence for initiation of translation in vertebrates (Kozak, 1987). The 30 hydrophobic amino acids at the N-terminus may serve as signal peptide. Mature DuIFN thus seems to consist of 161 amino acids. Sequence comparison with ChIFN revealed 50% identity at the amino acid level (Fig.

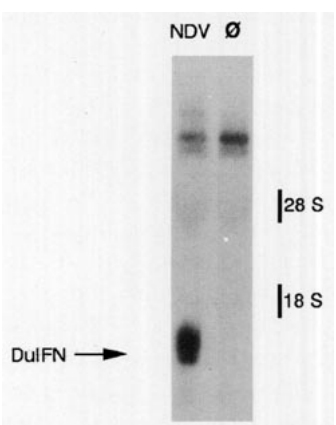

FIG. 2. DuIFN transcripts in virus-induced duck embryo cells. Cells were treated for 8 hr with UV-inactivated Newcastle disease virus (NDV) or were left untreated (Ø) before total RNA was prepared and subjected to Northern blot analysis with a radiolabeled fragment of the DuIFN gene.

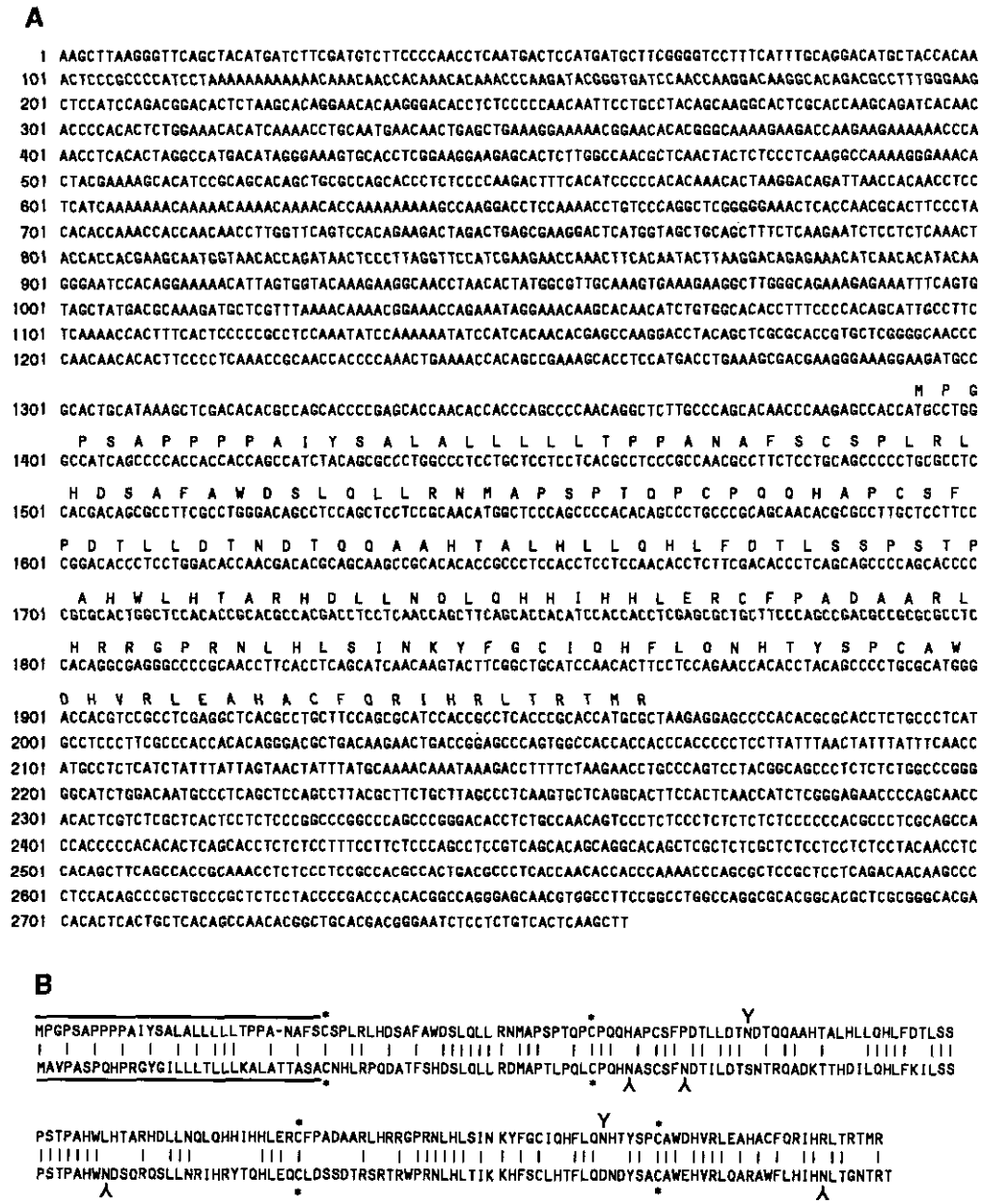

FIG. 3. Sequence of a genomic fragment of duck DNA containing an intronless IFN gene. (A) Complete nucleotide sequence of a 2.7-kb *Hin*dIII fragment that contains a gene for DuIFN. The deduced amino acid sequence is given in single-letter code. (B) Comparison of the amino acid sequences of DuIFN (top) and ChIFN (bottom). Lines mark the putative signal peptides of the two proteins. Cysteine residues conserved in mammalian IFNs-α, ω, and τ are highlighted by asterisks. Potential N-glycosylation sites are indicated by fork-like structures.

3B). Identity at the nucleotide level was 73%. Although the homology to mammalian type I IFNs is low (about 20% or less identity at the amino acid level), the critical cysteine residues (Sekellick *et al.*, 1994) are conserved (Fig. 3B). DuIFN has two potential N-glycosylation sites, suggesting that it is a glycoprotein like ChIFN (Schultz *et al.*, 1995b) and some mammalian type I IFNs (Weissmann and Weber, 1986).

First direct evidence that the open reading frame of the cloned DNA codes for DuIFN came from expression experiments in transfected COS7 cells. Supernatants of such cells contained an activity that protected duck embryo cells from destruction by VSV. Quantification with the test system described below demonstrated that the 72-hr supernatants of transfected COS cells contained about 25,000 units of DuIFN per milliliter, whereas supernatants of COS cells transfected with the empty vector contained no such activity.

DuIFN produced in *E. coli* is biologically active

Assuming that the cysteine residue at position 31 is the first amino acid of mature DuIFN (Fig. 3B), we ampli-

fied the downstream coding region of the DuIFN gene by PCR and cloned the product into the prokaryotic expression vector pET3a. Expression of this construct should yield recombinant DuIFN lacking a signal peptide. Since DuIFN accumulated in bacterial inclusion bodies, it could easily be collected from the cell lysate by centrifugation. About 10% of the recombinant protein could be recovered after solubilization of the pellet with 6 *M* guanidine hydrochloride and exhaustive dialysis. The remaining part of DuIFN was lost due to precipitation during the dialysis step. DuIFN in the soluble fraction was about 50% pure. After chromatography on a Q-Sepharose column followed by a MonoS column, DuIFN was at least 95% pure (data not shown). Due to additional losses during chromatography, this purification procedure yielded only about 10 μg of purified DuIFN per liter of *E. coli* culture.

To increase the yields of recombinant DuIFN, we tested whether the solubility of DuIFN could be enhanced. The yields improved when the inclusion bodies were solubilized in buffer containing 0.3% Triton X-100 and when a higher Tris–HCl concentration was used. When the detergent was also present during the dialysis step, up to 60% of DuIFN remained in solution (Fig. 4, lane 3). DuIFN was virtually pure after chromatography on Q-Sepharose and Fractogel EMD SO_3^- columns (Fig. 4, lanes 4 and 5). This purification protocol yielded up to 500 μg of purified DuIFN per liter of *E. coli* culture. However, its specific activity was significantly reduced compared to the DuIFN purified according to the former protocol.

The best results were achieved when DuIFN was applied to Ni^{2+} chelate agarose chromatography under denaturing conditions and when the elution was performed

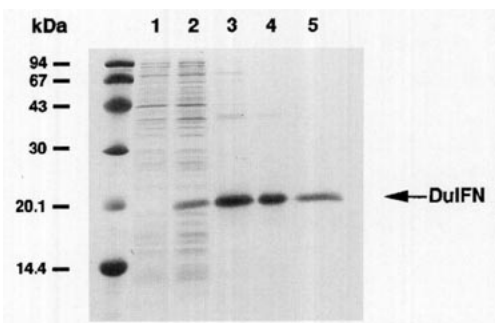

FIG. 4. Purification of recombinant DuIFN from *E. coli*. Samples from the various steps of the protein purification procedure were analyzed by electrophoresis through a 18% SDS–polyacrylamide gel and staining with Coomassie blue. Total cell lysates of bacteria transformed with the DuIFN expression construct were analyzed before induction (lane 1) or after induction with IPTG (lane 2). After solubilization with 6 *M* guanidine hydrochloride, DuIFN was the predominant protein in the preparation (lane 3). DuIFN in the flowthrough of a Q-Sepharose column was about 90% pure (lane 4). DuIFN could be separated from contaminating proteins by a fractogel column (lane 5). The molecular mass of marker proteins is shown. The gel position of DuIFN is indicated by an arrow.

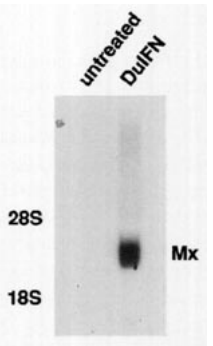

FIG. 5. Mx gene induction in duck embryo cells by recombinant DuIFN. Cells were treated for 6 hr with 10 ng (1000 units) of recombinant DuIFN per milliliter before total RNA was prepared and samples of 20 μg were subjected to Northern blot analysis. The membrane was probed with radiolabeled duck Mx cDNA.

in the presence of urea at low pH. Using this protocol we obtained more than 1 mg of DuIFN per liter of *E. coli* culture. This material was about 90% pure and showed high specific activity (data not shown).

When used at 10 ng per milliliter, DuIFN purified according to the first protocol activated the IFN-inducible Mx gene of duck embryo cells (Fig. 5) and protected these cells against destruction by VSV. Since an international standard for DuIFN is not available, the specific activity of DuIFN could not be determined accurately. We defined one unit of DuIFN as the dose which protected a duck embryo cell line (ATCC CCL141; DEC141) from destruction by VSV under the conditions described under Material and Methods. Using this definition, recombinant DuIFN purified according to the first and third protocols exhibited a specific antiviral activity of about 10^8 units per milligram.

To determine the inhibitory effect of recombinant DuIFN on the replication of various viruses in DEC141 cells, we treated the cultures for 15 hr with either 10 or 100 units of purified DuIFN per milliliter before virus challenge. Infection experiments with VSV showed that the viral titers were dramatically reduced in the supernatants of IFN-treated cultures at 12 and 48 hr postinfection (Table 1). The protective effect of DuIFN against influenza A virus and NDV was less pronounced: the influenza virus titers were 10- to 100-fold reduced in the supernatants of IFN-treated cells (Table 1). The yields of infectious NDV from IFN-treated cultures were 20- to 40-fold reduced at 12 hr postinfection (Table 1), and the reduction was only 5- to 10-fold at 48 hr postinfection, indicating that the protective effect against this virus was transient.

DuIFN inhibits the replication of DHBV in primary duck hepatocytes

To test whether DuIFN can protect primary duck hepatocytes from DHBV infection, the cultures were treated with 100 units per milliliter of DuIFN for 15 hr before

TABLE 1

Antiviral Activity of Recombinant DuIFN

Virus	IFN concentration (U/ml)	Virus yields in the supernatants of DuIFN-treated DEC cells ($TCID_{50}$/ml) 12 hr	48 hr
VSV	Plain medium	6.8×10^5	1.5×10^7
	10	1.5×10^2	3.2×10^2
	100	<10	<10
NDV	Plain medium	1.5×10^5	1.5×10^5
	10	3.2×10^3	1.5×10^4
	100	6.8×10^3	3.2×10^4
Flu	Plain medium	6.8×10^4	3.2×10^6
	10	3.2×10^3	3.2×10^4
	100	6.8×10^3	3.2×10^4

infection. The virus inoculum was removed after 5 hr, and fresh medium with or without DuIFN was added. Cell lysates were prepared 5, 24, and 48 hr after virus was added and examined for the presence of viral DNA by PCR using primer pairs that can discriminate between cccDNA and other forms of viral DNA. Cells harvested 5 hr after virus infection should not contain detectable amounts of replication-competent viral cccDNA, although they are expected to contain significant amounts of other forms of viral DNA, mainly due to the inoculum that could not be removed during the washing procedure. As expected, the primers designed to selectively detect cccDNA yielded no PCR products, whereas other viral DNA forms were present at a high level (Fig. 6A, lane 3). Since both primers were equally efficient on DHBV plasmid DNA (Fig. 6A, lane 1), this result confirmed the high selectivity of the selected primer pairs for the various forms of viral DNA, as previously described (Köck and Schlicht, 1993). At 24 hr postinfection low levels of viral cccDNA were observed in both the IFN-treated and the untreated cultures (Fig. 6A, lanes 4–6), indicating that penetration of DHBV and conversion of its genome into the cccDNA form were not blocked in IFN-treated cells. In the untreated control culture, the viral cccDNA level increased several fold between 24 and 48 hr postinfection (Fig. 6, lane 7), whereas it remained low in cultures treated with DuIFN (Fig. 6, lanes 8 and 9). Analysis of total DNA also reflected the inhibitory effect of DuIFN, although the high background of the virus inoculum complicated the evaluation of total viral DNA levels. The concentration of total viral DNA increased several fold in untreated cultures between 24 and 48 hr postinfection, but remained at baseline levels in the IFN-treated cultures (Fig. 6B). It thus seemed that DuIFN can block DHBV infection in primary duck hepatocytes and that amplification of the viral genome represents the target of IFN action. This view is supported by the finding that the culture kept in the continuous presence of DuIFN contained slightly less viral cccDNA than the culture that was pretreated with but not maintained in IFN containing medium (Fig. 6A, lane 9).

We next examined whether DuIFN was also effective when added after the cells were infected with DHBV. At 24 hr post-virus infection, treatment of primary hepatocyte cultures was started with either 100 units per milliliter of DuIFN or with 20 μg per milliliter of the nucleoside analog ddG, which is known to block DHBV replication very effectively (Köck and Schlicht, 1993). At various times after the onset of treatment, cell lysates were prepared and analyzed for viral DNA content by PCR using primers which selectively amplify viral cccDNA. DuIFN and ddG were similarly effective inhibitors of DHBV under these conditions (Fig. 7). DHBV cccDNA levels stopped increasing shortly after the onset of IFN treatment and started to decrease slowly. After 14 days of treatment with DuIFN, the viral cccDNA had reached a very low level in the infected cultures (Fig. 7). When the IFN treatment was started at 72 hr postinfection, the inhibitory effect was less pronounced at early times but was similarly strong at later times (data not shown). The kinetics of viral cccDNA clearance induced by DuIFN and ddG were almost the same (Fig. 7). No morphological differences and no differences in protein synthesis rates were observed between IFN-treated and nontreated hepatocyte cultures (data not shown), indicating that our IFN preparations were not toxic.

DISCUSSION

By using the recently cloned chicken IFN cDNA (Sekellick *et al.*, 1994) as a hybridization probe, we were able

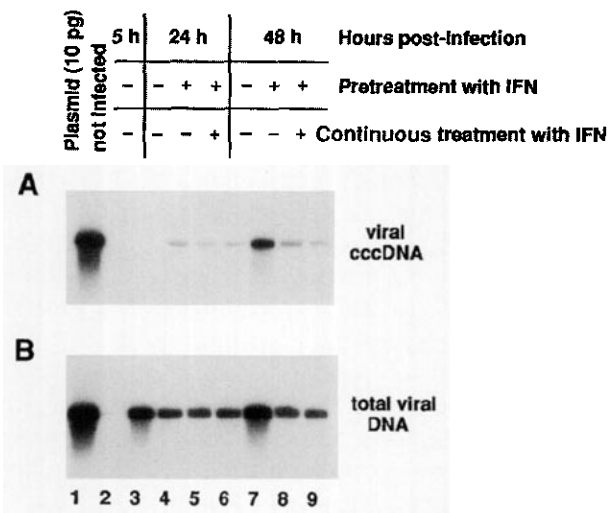

FIG. 6. Influence of DuIFN on the susceptibility of primary duck hepatocytes to DHBV. Cells were incubated with 100 U/ml of DuIFN for 15 hr prior to infection with DHBV. Cells were lysed at the indicated times after infection and samples of the lysates where subjected to PCR analysis with primer pairs that permit selective amplification of viral cccDNA (A) or amplification of total viral DNA (B). The PCR products were analyzed by Southern blotting.

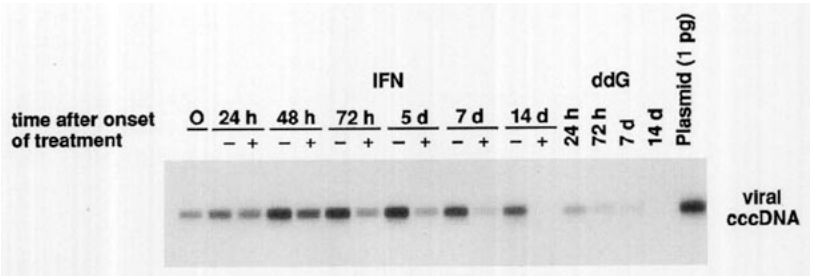

FIG. 7. Therapeutic effect of DuIFN on primary duck hepatocytes infected with DHBV. Treatment of DHBV-infected cells with 100 U/ml of DuIFN or 20 μg/ml of ddG was started at 24 hr postinfection. At the beginning of the treatment (0 hr) and at the indicated times after the onset, the cells were harvested and samples of the lysates were subjected to PCR analysis with a primer pair that permits selective amplification of viral cccDNA. The PCR products were analyzed by Southern blotting. The symbol (−) indicates untreated control cultures; the symbol (+) indicates cultures kept in the continuous presence of DuIFN.

to isolate a DNA fragment that contained a gene for DuIFN. The cloned DuIFN is most likely a type I IFN for several reasons: (i) it is encoded by an intronless gene like the mammalian genes for IFNs-α, β, and ω (Weissmann and Weber, 1986), (ii) it is strongly induced in response to virus infection, (iii) it shows conservation of four cysteine residues which are present in mammalian IFNs-α, ω, and τ (Sekellick *et al.*, 1994), and (iv) it is a potent inducer of the Mx gene which is strongly induced by type I but not type II IFN in mammals (Aebi *et al.*, 1989). DuIFN is 73% identical to chicken IFN at the nucleotide level and 50% identical at the amino acid level. The chicken has more than 20 IFN genes (C. Sick and U. Schultz, unpublished results) which have not yet been classified. It remains unknown whether the cloned DuIFN should be regarded as an IFN-α, β, or ω.

Expression of a polypeptide in *E. coli* that corresponds to mature DuIFN yielded antivirally active material that could be purified to homogeneity. A major difficulty in this regard is the low solubility of recombinant DuIFN. After solubilization in 6 *M* guanidine hydrochloride, recombinant DuIFN showed a high tendency to precipitate upon dialysis against physiological buffers. The material that remained soluble exhibited high specific antiviral activity, indicating that correct refolding of DuIFN had indeed occurred. Nonionic detergents increased the solubility of DuIFN but, unfortunately, strongly reduced its biological activity. The protocol currently used which includes Ni^{2+} chelate agarose chromatography seems to be the method of choice to obtain high yields of DuIFN of sufficient purity and specific activity for future *in vivo* studies.

Recombinant DuIFN protected duck fibroblasts very effectively from destruction by lytic RNA viruses and further showed a strong inhibitory effect on DHBV in infected primary duck hepatocytes. The latter result is of great value because it demonstrates that DuIFN may serve as a new reagent for investigating the inhibitory effect on IFNs toward hepadnaviruses. Adding DuIFN to the hepatocyte cultures soon after infection with DHBV blocked the accumulation of the replicative form of viral DNA about as effectively as addition of the nucleoside analog ddG. Continuous incubation of the infected hepatocytes with IFN had a pronounced curative effect: viral cccDNA disappeared from the cultures with kinetics which resembled that in ddG-treated cultures. Due to the complex nature of this process (Nassal and Schaller, 1993), inhibition could result from blocking either synthesis, packaging, or reverse transcription of the pregenomic viral RNA. Alternatively, IFN may inhibit viral protein synthesis. It is unlikely that DuIFN has an inhibitory effect on the early steps of DHBV infection, since pretreatment of the hepatocytes with DuIFN did not abolish the initial appearance of viral cccDNA in the infected cells. Further experiments should allow unambiguous identification of the IFN-susceptible replication steps of DHBV.

The results of our experiments with DHBV-infected hepatocyte cultures suggest that DuIFN may be useful for therapy studies in chronically infected ducks. Since sufficient quantities of active DuIFN can be produced in *E. coli*, such experiments now become feasible. Systematic *in vivo* studies may stimulate the rational design of new combination therapies for the treatment of the human diseases associated with chronic HBV infection.

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REFERENCES

Aebi, M., Fah, J., Hurt, N., Samuel, C. E., Thomis, D., Bazzigher, L., Pavlovic, J., Haller, O., and Staeheli, P. (1989). cDNA structures and regulation of two interferon-induced human Mx proteins. *Mol. Cell. Biol.* **9**, 5062–5072.

Ausubel, F., Brent, R., Kingston, R., Moore, D., Seidman, J., Smith, J., and Struhl, K. (1992). "Current Protocols in Molecular Biology." Wiley, New York.

Bazzigher, L., Pavlovic, J., Haller, O., and Staeheli, P. (1992). Mx genes show weaker primary response to virus than other interferon-regulated genes. *Virology* **186**, 154–160.

Chomczynski, P., and Sacchi, N. (1987). Single-step method of RNA

isolation by acid guanidinium thiocyanate–phenol–chloroform extraction. *Anal. Biochem.* **162,** 156–159.

Hoofnagle, J. H. (1992). "Interferon. Principles and Medical Applications" (S. Baron, D. H. Coppenhaver, F. Dianzani, W. R. Fleischmann, T. K. Hughes, G. R. Klingsel, D. W. Niesel, G. J. Stanton, and S. K. Tyring, Eds.), 2nd ed., pp. 433–462. University of Texas Medical Branch of Galveston, Galveston, TX.

Isaacs, A., and Lindenmann, J. (1957). Virus interference. I. The interferon. *Proc. R. Soc. Lond.* **147,** 258–267.

Israel, J., and London, W. T. (1991). Liver structure, function, and anatomy: Effects of hepatits B virus. *Curr. Top. Microbiol. Immunol.* **168,** 1–20.

Kaaden, O. R., Lange, S., and Stiburek, B. (1982). Establishment and characterization of chicken embryo fibroblast clone LSCC-H32. *In Vitro* **18,** 827–834.

Kozak, M. (1987). An analysis of 5′-noncoding sequences from 699 vertebrate messenger RNAs. *Nucleic Acids Res.* **15,** 8125–8148.

Köck, J., and Schlicht, H.-J. (1993). Analysis of the earliest steps of hepadnavirus replication: Genome repair after infectious entry into hepatocytes does not depend on viral polymerase activity. *J. Virol.* **67,** 4867–4874.

Mason, W. S., Seal, G., and Summers, J. (1980). Virus of Pekin ducks with structure and biological relatedness to human hepatitis B virus. *J. Virol.* **36,** 829–836.

Nassal, M., and Schaller, H. (1993). Hepatits B virus replication. *Trends Microbiol.* **1,** 221–228.

Pavlovic, J., Zurcher, T., Haller, O., and Staeheli, P. (1990). Resistance to influenza virus and vesicular stomatitis virus conferred by expression of human MxA protein. *J. Virol.* **64,** 3370–3375.

Perrillo, R. P., Schiff, E. R., Davis, G. L., Bodenheimer, H. C., Lindsay, K., Payne, J., Dienstag, J. L., OtBrian, C., Tamburro, C., Jacobson, I. M., Sampliner, R., Feit, D., Lefkowitch, J., Kuhns, M., Meschievitz, C., Sanghvi, B., Albrecht, J., and Gibas, A. (1990). A randomized, controlled trial of interferon alfa-2b alone and after prednisone withdrawal for the treatment of chronic hepatitis B. *N. Engl. J. Med.* **323,** 295–301.

Saracco, G., Rizzetto, M., and Verme, G. (1994). Interferon in chronic hepatitis B. *Antiviral. Res.* **24,** 137–143.

Schödel, F., Weimer, T., Fernholz, D., Schneider, R., Sprengel, R., Wildner, G., and Will, H. (1991). "Molecular Biology of the Hepatitis B Virus" (A. McLachlan, Ed.), pp. 53–80. CRC Press, Boca Raton, FL.

Schultz, U., Kaspers, B., Rinderle, C., Sekellick, M. J., Marcus, P. I., and Staeheli, P. (1995a). Recombinant chicken interferon lacks intrinsic macrophage activating factor activity. *Eur. J. Immunol.* **25,** 847–851.

Schultz, U., Rinderle, C., Sekellick, M. J., Marcus, P. I., and Staeheli, P. (1995b). Recombinant chicken interferon from Escherichia coli and transfected COS cells is biologically active. *Eur. J. Biochem.* **229,** 73–76.

Sekellick, M. J., Ferrandino, A. F., Hopkins, D. A., and Marcus, P. I. (1994). Chicken interferon gene: Cloning, expression and analysis. *J. Interferon Res.* **14,** 71–79.

Studier, F., and Moffat, B. (1986). Use of bacteriophage T7 RNA polymerase to direct selective high-level expression of cloned genes. *J. Mol. Biol.* **189,** 113–130.

Tuttleman, J. S., Pugh, J. C., and Summers, J. W. (1986). In vitro experimental infection of primary duck hepatocyte cultures with duck hepatitis B virus. *J. Virol.* **58,** 17–25.

Weissmann, C., and Weber, H. (1986). The interferon genes. *Prog. Nucleic Acid. Res. Mol. Biol.* **33,** 251–300.

Ziegler, R. E., and Joklik, W. K. (1981). Effect of interferon on multiplication of avian sarcoma virus B77 in duck embryo fibroblasts. *J. Interferon Res.* 1, 521–537.